Characterization of Colonic Macrophages in Mice Exposed to Social Stressor During Oral Challenge with *Citrobacter rodentium*.

Honors Research Thesis

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Abstract: Chronic colonic inflammation, as well as exposure to psychological stressors, are well known risk factors for the development of colorectal cancer. Whether these two predisposing factors are linked, however, is not known. A murine social stressor, called social disruption (SDR), was used to test whether stressor exposure would increase colonic inflammation in mice infected with the colonic pathogen *Citrobacter rodentium*. When compared to non-stressed control mice infected with *C. rodentium*, stressor-exposure significantly increased pathogen-induced colonic histopathology, inflammatory cytokine gene expression, and the accumulation of $F4/80^{+}$ macrophages in the colon. Other studies demonstrate that exposure to the SDR stressor without infection significantly increases the number of inflammatory monocytes in the spleen. Because splenic inflammatory monocytes can traffic to sites of inflammation, the goal of this study was to determine whether these accumulated colonic F4/80+ macrophages display an inflammatory phenotype (i.e., $L6C^{hi}CCR2^{+}CD11b^{+}$) by using flow cytometry. To test whether colonic inflammation could be significantly enhanced by colonic macrophages, an adoptive transfer model was also used. In this experiment, monocytes isolated from the spleens of stressed mice were introduced into naïve mice, which were later infected with *C. rodentium*. It is expected that these non-stressed recipient mice will display similar inflammation and pathology as stressed mice. If proven true, it would mean that the stressor-primed splenic inflammatory monocytes are responsible for enhanced colonic inflammation. Increases in colonic macrophages may account for stressor-induced increases in colonic inflammation. Future studies will test the hypothesis that this increased inflammation predisposes animals to developing colorectal cancer.

1. Introduction

Psychological stress has been shown to have profound effects on physiological functions, especially in disease pathology (Bartolomucci *et al.*, 2007). Importantly, among the different functions that are affected is the immune system. Stress can have a complex effect on the immune system, in some situations enhancing it, and others depressing it; however in most cases the effects are deleterious. Many of these negative effects are due to enhancement of the inflammatory immune response, which can lead to chronic and pathological inflammation. Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease are particularly sensitive to stress, being linked to causing new flare-ups. Furthermore, chronic inflammation and stress have been implicated in increased risk for developing cancer, especially in the colon.

1.1 Psychological Stress:

Psychological stress can be defined as an organism's physiological and behavioral response to a perceived potential danger. Stress alters and modulates many of the body's normal functions in order to prepare the organism to cope with the potential danger, through a "fight or flight" response. When an organism encounters a stressor, two major pathways are activated which bring about these modulations (Bartolomucci, 2007). One of these pathways involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis and the release of glucocorticoids (Leonard *et al.*, 2005). Glucocorticoids (cortisol in humans, corticosterone in mice) stimulate an increase in gluconeogenesis to ensure high glucose availability for ready use in quick bursts of energy (Khani *et al.*, 2001). Additionally, glucocorticoids generally have immunosuppressive activity and are effective in reducing inflammation (Coutinho, 2011). The second major system

is the sympathetic nervous system (SNS), which innervates almost every organ in the body and is responsible for the well known "fight or flight" response (Bartolomucci, 2007). Activation of the SNS results in the almost immediate release of catecholamines such as epinephrine and norepinephrine (Carrasco *et al.*, 2003). Catecholamines have many immunomodulatory behaviors, mainly regulatory and suppressive roles, such as decreasing TNF-α production by macrophages (Nance *et al.*, 2007).

1.2 Stress Effect on the Immune System:

There is a widely acknowledged intimate relationship between stress and the immune system (Bartolomucci, 2007), (Pruett *et al.*, 2003), (Segerstrom *et al.*, 2004). The relationship is bi-directional: stress is capable of modulating the immune system, and the immune system is capable of inducing a stress response. Generally, stress is known to suppress the immune system. The release of glucocorticoids has strong immunosuppressive and anti-inflammatory effects, and they are known to inhibit the activity of lymphocytes and macrophages (Da Silva *et al.*, 1999), (Reiche, 2004). However in many cases, stress has been shown to enhance the immune response, especially in inflammatory and autoimmune diseases (Dhabhar *et al.*, 2002), in part by causing a redistribution of immune cells throughout tissues and the blood (Dhabhar *et al.*, 2012).

Social stress in particular is a well defined and effective laboratory stressor (Bartolomucci, 2007). Venzala *et al.* examined the comparability of murine social stressors as models for human psychological disorders. They found that their stressor models significantly increased anxiety-like and other abnormal behavior over a range of behavior tests. Although they concluded social stressors are not perfect models for clinical depression, they acknowledged that

they accurately model many of the symptoms and behaviors of depression and anxiety-related disorders (Venzala *et al.*, 2012).

Social disruption (SDR) is a well defined social stressor in which subordinate young mice are attacked and defeated by an older aggressive mouse over a span of 2 hours for 6 consecutive days. Defeated mice have been shown to have elevated levels of systemic corticosterone as well as modulated behavior (Avitsur *et al.*, 2001). The SNS is also activated during SDR, as are brain regions associated with the activation of the SNS. Importantly, it has been reported that SDR can increase $CD11b^{+}$ myeloid-derived leukocytes, such as neutrophils and monocytes both in circulation and in the spleen (Engler *et al.*, 2004). Furthermore, splenocytes isolated from these stressed mice have been shown to have a decreased sensitivity to glucocorticoid inhibition in addition to expressing higher levels of inflammatory cytokines upon antigen stimulation (Bailey *et al.*, 2003).

1.3 Inflammation

Inflammation is marked by the release of cytokines that stimulate the symptoms that are associated with the inflammatory response. Two well known markers are TNF-α and IL-1. TNF-α is important in basic immunity against pathogens including bacterial, viral and parasitic infections. TNF-α is produced primarily by activated macrophages and T cells. It precipitates the activation of complex and numerous pathways that culminate in the activation of NF-κB, a transcription factor pathway which has been shown to be pivotal in 221 other molecular associations (Bouwmeester *et al.*, 2004). TNF-α stimulates the production of different selectins on vascular endothelial cells as well as vasodilatation, both of which are essential for recruiting leukocytes out of circulation and into the tissue. TNF-inhibiting drugs effectively treat a wide

range of chronic inflammatory diseases (Vincent *et al.*, 2012) including Crohn's disease (Hanauer *et al.*, 2002). TNF antibodies can also prevent septic shock when injected with a lethal dose of bacteria (Tracey *et al.*, 1987).

There are two forms of interleukin-1, IL-1α and IL-1β, that play important roles in the inflammatory response and inducing the expression of many other inflammatory genes (Dinarello *et al.*, 2009). Exposure to the SDR stressor has been shown to lead to increases in both TNF-α and IL-1 upon stimulation (Engler *et al.*, 2004).

Chemokines are a subclass of cytokines known for their role in leukocyte chemotaxis and homing. Chemokines are produced by cells at the site of infection, and incoming leukocytes travel up the concentration gradient towards the site. They can also be used to concentrate immune cells in specific locations, such as lymphoid tissue (Bachmann *et al.*, 2006). CCL2 is an important inflammatory cytokine. It can be secreted by a range of cells including monocytes, macrophages, T cells, fibroblasts, endothelial cells, and mast cells. The cell receptor for CCL2 is CCR2, and is commonly found on proinflammatory cells such as infiltrating monocytes (Shachar *et al.*, 2013). CCR2 is essential for monocyte recruitment and K/O mice exhibit significantly reduced numbers of leukocytes in tissue (Kuziel *et al.*, 1997).

Inflammation in the colon is of particular interest because of its association with inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, and its predisposal for developing colorectal cancer (CRC). Furthermore, colonic inflammation has strong correlations with stress.

The lumen of the colon is home to approximately ten times more bacterial cells than there are human cells in the body. These bacteria are commonly referred to as the colonic microbiota and have been shown to have numerous beneficial effects for the host. The epithelium of the

colon provides a physical barrier to contain the bacteria both through tight junctions and the production of a mucosal layer. The lamina propria, which lies directly beneath the epithelial layer, contains a wide variety of immune cells including macrophages, neutrophils, dendritic cells, natural killer T cells, and T and B cells. There is an intricate balance between these immune cells and the microbiota. The immune cells must be prevented from activating against all the normal microbiota, but also must be able to recognize and attack pathogens. Dysfunction of this balance can lead to chronic inflammation in the colon, which can lead to pain, discomfort, tissue pathology, and potentially the removal of the colon or development of CRC (Abraham *et al.*, 2009). One type of cell that is involved with chronic inflammation in the colon it the macrophage.

1.4 Macrophages

Macrophages are generally tissue associated leukocytes of the innate immune response. They are mononuclear phagocytes, and play a vital role in orchestrating the initial immune and inflammatory responses. Monocytes are myeloid-derived cells in the blood stream, which differentiate into macrophages upon leaving the blood and entering the tissue.

Both monocytes and macrophages are highly heterogenous, and display a wide range of phenotypes based on their tissue location and function (Gordon and Taylor, 2005). Different macrophage phenotypes can have diverse and contradictory behaviors including phagocytic, inflammation-promoting, inflammation suppressing, cancer-promoting, cancer-suppressing or wound healing. F4/80 is a cell surface marker that is especially prevalent and highly expressed on macrophages in tissue, but lower levels can be found on dendritic cells and activated eosinphils. It is commonly used as a marker for macrophages in the lamina propria, where it is

highly expressed (Gordon *et al.*, 2011). Important for the colon are inflammatory and anti-inflammatory macrophages, often roughly referred to as M1 and M2, respectively. The M2 phenotype is associated with wound healing, tissue restructuring and anti-inflammatory behavior. Arginase, an enzyme which competitively inhibits iNOS activity, and TGF-β production are associated with M2 phenotypes (Martinez *et al.*, 2008). The M1 phenotype is associated with inflammation and marked by TNF-α, iNOS and CCL2 production. TNF-α plays a crucial role in recruiting monocytes/macrophages to sites of infection, inflammation and tissue damage, as well as organizing cellular structure (Locksley *et al.*, 2001). Although TNF-α can have anti-tumor properties, over-expression can lead to cancer through a number of pathways, including growth and angiogenic factors, cellular restructuring, and most importantly for this study, through inflammation, recruitment of macrophages and stimulation of iNOS (Szlosarek *et al.,* 2001). The enzyme iNOS is expressed by inflammatory macrophages and leads to the synthesis of nitric oxide (NO). NO and its byproducts can undergo a number of disruptive reactions with DNA, many leading to cell death, but some leading to genetic mutations (Ambs, *et al.*, 1998). Tumors are associated with high levels of iNOS, and inhibition of iNOS has been shown to significantly decrease adenocarcinoma development in the pancreas of hamsters (Takahashi *et al.*, 2008), as well as in an azoxymethane-induced model for CRC in rats (Takahashi *et al.*, 2006).

1.5 Citrobacter rodentium

Citrobacter rodentium is a noninvasive murine pathogen which colonizes the epithelium of the colon and induces disease pathology similar to enteropathogenic *Escherichia coli* (EPEC) in humans. Previously classified as *Citrobacter freundii* biotype 4280, *C. rodentium* strains were shown to contain genes identical to eaeA and eaeB in *E. coli*, which are necessary for the

characteristic attaching and effacing lesions associated with EPEC infections (Schauer *et al.*, 1995). Oral infections with *C. rodentium* result in a well defined disease known as transmissible colonic hyperplasia which is characterized by thickening of the mucosal layer, epithelial cell proliferation, goblet cell depletion and elongation of intestinal crypts (Bhinder *et al.*, 2013), (Higgins *et al.*, 1999).

Importantly, infections with *C. rodentium* have been shown to be effective models for inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease (Higgins *et al.*, 1999). Higgins *et al.* demonstrated that the immune response to *C. rodentium* was marked by high percentages of $CD4^+$ T-cells, increased IL-12, and TNF-α; all of which indicate a Th1 response. They also noted that the infiltration patterns of the T-cells and macrophages bore strong resemblance to the patterns seen in the chronic inflammation of IBDs.

1.6 Cancer

The intricate relationship between stress, the immune system and inflammation have important implications for cancer growth and development. Understanding these relationships is vital for forming an effective cancer prevention and treatment regimen.

Psychological stress and other behavioral factors have been shown to play an important role in cancer development and progression (Lutgendorf and Sood, 2011), (Costanzo *et al.*, 2011). Stress has been linked to shorter survival in breast cancer patients (Giese-Davis and Spiegel, 2003). Exposing laboratory mice to experimental psychological stressors has been used to model these effects in humans, and in a murine breast cancer model, exposure to stress increased cancer metastasis by 30-fold. This effect was found to be due to stress induced activation of the sympathetic nervous system (Sloan *et al.*, 2010). These effects, however, are not

limited to breast cancer. Stressed mice have also been shown to develop skin cancer significantly quicker than non-stress mice in an ultraviolet light-induced squamous cell carcinoma model (Saul *et al.,* 2005). The mechanisms by which exposure to psychological stressors can enhance cancer progression are not well defined, but could involve stressor-induced increases in inflammatory responses.

Several studies have demonstrated a key relationship between chronic inflammation and cancer growth and development (Colotta *et al.,* 2009). This effect is likely bidirectional. Inflammatory genes have been shown to be induced by oncogenes. For example, in an *in vitro* thyroid cancer cell model, the *RET/PTC1* oncogene induces the expression of inflammatory genes, such as the gene encoding for CCL2 (Borrello *et al.,* 2005). CCL2 recruits inflammatory monocytes, as well as activated T cells, to sites of inflammation where they produce inflammatory cytokines, like TNF-α and IL-1β, as well as reactive oxygen and nitrogen intermediates (Daly *et al.,* 2003). These inflammatory cytokines and reactive intermediates have been shown to promote tumorigenesis through induction of a pathway that inhibits DNA mismatch repair enzymes and increases angiogenic factors (Jung *et al.,* 2004) (Koshiji *et al.,* 2005). While the cancer-inflammation relationship is important for all cancer types, it is especially apparent in colorectal cancer. Diseases marked by chronic colonic inflammation, such as inflammatory bowel diseases (IBD), have been shown to greatly increase the likelihood of developing colorectal cancer (CRC) (Jawad *et al.,* 2011). It is estimated that only 20% of CRC cases are associated with hereditary causes (Rustgi, 2007), while the remaining portions are induced by environmental factors, and overwhelmingly those which induce inflammation and colitis (Terzic, *et al.,* 2010). A collation of data from 19 articles suggests that 30 years after diagnosis with IBD, an average of 1 in 5 cases will result in CRC diagnosis, and about 50% of

those cases result in death (Lakatos *et al.,* 2008). Importantly, the general risk of developing CRC can be significantly reduced through regular use of anti-inflammatory drugs such as aspirin (Chan *et al.,* 2007). Thus, it is evident that colonic inflammation significantly increases CRC progression.

M1 macrophages create an inflammatory environment ideal for tumor formation as previously described, while M2 is the phenotype expressed by tumor-associated macrophages (TAM) (42). Tumors have been shown to produce CCL2, a chemotactic cytokine for CCR2+ inflammatory macrophages (Sica *et al.,* 2008). Thus, stressor-induced increases in colonic macrophages, particularly during chronic colonic inflammation, could result in an internal environment conducive to the formation of colorectal cancer.

Purpose: The purpose of this thesis was to characterize macrophages in the colon during stressor-enhanced pathogen-induced inflammation, and to test the hypothesis that cytokines associated with M1 macrophages will be increased in stressor-exposed mice. Another goal of this thesis examine if social stress and colonic inflammation increases susceptibility to developing CRC.

3. Methods and Materials

3.1 Mice and Social Disruption

Wild type, male C57BL/6 mice, aged 6-8 weeks, were purchased from Charles River Laboratories (Wilmington, MA) and housed 3 per cage in an AAALAC-approved facility. Mice were kept on a 12 hr light:dark schedule with lights on at 0600 hrs and given food and water *ad libitum.* After arrival, mice were given one week for acclimation, and observed for any sign of

inter-cage aggression. Social disruption involves introducing an aggressive male mouse into the cage of the SDR mice for two hours per evening, from 1700 to 1900 hrs, for six consecutive evenings. During this time, the aggressive male attacks and repeatedly defeats the young mice. SDR cages were observed for 20 min at the beginning of each cycle to ensure adequate fighting. Control mice were left undisturbed, and monitored for inter-cage fighting. After the first cycle of SDR, all mice were infected via oral gavages with 100 µl of *Citrobacter rodentium* strain DBS120 (pCRP1::Tn5) in PBS at a concentration of about 1-3 x 10^7 CFU/ml. Mice were sacrificed on days 0, 6, 12, and 24 post-infection by CO_2 inhalation. Colons and spleens were removed and weighed, and snap-frozen in liquid nitrogen.

On days 1, 3, 6, 12, and 24 post-infection, stool was collected and cultured to determine extent of *C. rodentium* infection. Stool was homogenized and serially diluted in PBS. The *C. rodentium* used had a kanamycin resistance cassette, and therefore aliquots of the homogenized stool were plated on Lactose MacConkey agar (Becton Dickinson) supplemented with kanamycin (40 µg/ml) to select for the pathogen. Plates were incubated at 37 °C overnight without CO2, and the number of colony forming units (CFU) per gram of fecal matter determined.

3.2 Measurement of gene expression by Semi-Quantitative Real Time PCR Analysis

On days 0, 6, 12, and 24 post-infection, colons were harvested and total RNA was extracted using Trizol reagent as per manufacturer's protocol (Invitrogen, Carlsbad, CA). After isolation, total RNA was spectrophotometrically quantified so that a reverse transcription reaction of 2 µg of RNA could be performed to synthesize cDNA. The reverse transcription reaction makes a DNA copy (cDNA) of an RNA molecule, using the Avian Myeloblastosis Virus (AMV) Reverse Transcriptase enzyme (Promega Corporation, Madison, WI). These

samples then underwent Real-time Polymerase Chain Reaction (PCR), in order to amplify the nucleotide sequences of interest, which included mRNA for TNF-α, iNOS, CCL2, arginase, and TGF-β. The housekeeping gene that was used was 18S. Primers and probes were synthesized by Applied Biosystems.

The PCR mixture consisted of 2.5 µl of cDNA, 2.5 µl of primer mix, 2.5 µl of probe (or SYBR Green for arginase and TGF-β), 5 µl of sterile H_2O, and 12.5 µl of Tagman Universal Master Mix (PE Applied Biosystems, Foster City, CA) for a final volume of 25 µl. After an initial 2-min cycle at 50ºC followed by 10 min at 95ºC, the reaction ran for 40 total cycles, which consisted of a 15-s denaturing phase (90ºC) and a 1 min annealing/extension phase at 60ºC. The change in fluorescence was measured using an Applied Biosystems 7000 Prism Sequence Detector (PE Applied Biosystems) and analyzed using Sequence Detector version 1.0. The relative amount of transcript was determined using the comparative cycle threshold (C_t) method as described by the manufacturer.

3.3 Quantification of F4/80+ cells in colon tissue

Colons were harvested from SDR and HCC mice on days 0, 3, 6, 12, and 24 post infection and fixed in 10% formalin. Samples were then sent to the Vet Core where they were embedded in paraffin and sliced. The tissue was counterstained and treated with F4/80 primary antibody, and then a secondary antibody conjugated with horse radish peroxidase for visualization. The samples were photographed under a microscope at 100X. Every 5^{th} photograph was quantified using Photoshop, as described previously (Eubank *et al.*, 2009). In Photoshop, a positive pixel coloring was identified. The histogram was then run which turned all positive pixels black, and the rest of the pixels white. The percentage of pixels that were black was then determined. Pictures were cropped so that percentages reflected only colon tissue.

3.4 Lamina Propial Lymphocyte Isolation and Flow Cytometry

Colons were harvested from SDR and HCC mice on day 12 post infection and flushed with HBSS. Excess fat tissue was removed from the colon, bisected, and then cut into 0.5 cm pieces which were placed in 1 mM dithioerythritol in HBSS solution. Samples were then shaken at 37 ºC for 30 min, the supernatant removed, the tissue resuspended in 5% FBS in RPMI and collagenase (4.975 mg/25ml) solution, and shaken at 37ºC for an additional 90 min. Supernatant was then filtered with a 70 µl cell strainer, washed, counted, and resuspended for flow staining. Fifty thousand cells were stained with 1 µl of FITC labeled CD45, PerCP labeled F4/80, and APC labeled CD11b in panel one and CD45-FITC, CCR2-PE, and CD11b-APC in panel two. Two panels for lymphocytes were also used, with antibodies for CD45-FITC, CD3-PE, CD4-PerCP and CD8-APC. CD45 is a marker used to identify leukocytes in general. CD3 is a marker used to identify T cells, CD4 and CD8 are markers for helper and cytotoxic T cells. Expression of these cell markers was determined on a Becton-Dickinson FACSCaliber four color cytometer, and 10,000 events were recorded. Flow results were gated first for mononuclear cells via forward and side scatter, then CD45+ cells, then CD11b+ cells, then CCR2+CD11b+ cells. A gate was set on CD45+ cells to determine the percentage of mononuclear cells that were $CD45^+$. Within the $CD45^+$ cells, a gate was set to determine the percentage of $CD45^+$ cells that were $CD11b^+$, and what percentage of cells were both $CD11b^+$ and $CCR2^+$.

3.5 AOM Study

In a second study, mice subjected to SDR in combination with azoxymethane (AOM) to assess colonic tumor formation. One week after arrival, the mice were given an inter-peritoneal

(IP) injection of 100 µl of either AOM at a concentration 1 mg AOM per 1 kg of body weight, or PBS. The first cycle of SDR was started one week after IP injections and *C. rodentium* was gavaged afterwards. Five more cycles of SDR were performed, and then the mice were left undisturbed until 20 weeks post-IP injection. Body masses were measured every other week, starting on week 8. At week 20, mice were euthanized by CO_2 inhalation. Livers, spleens, and colons were removed and weighed, colon length was measured, and tissues were snap-frozen in liquid nitrogen. Before freezing, colons were examined for signs of tumors, contents were removed, and the tissue was bisected longitudinally into two equal pieces. Half was frozen for storage, and half was fixed in 10% formalin for hematoxylin and eosin (H&E) staining, and histopathology analysis.

3.6 Statistical Analysis

To determine the differences between SDR stressor mice and HCC control mice, two-factor analysis of variance (ANOVA) with group (i.e., HCC vs. SDR) and day (i.e., 0, 6, 12, and 24) as between subject variables. For studies involving AOM, a third factor was added (i.e. AOM vs. PBS). The level of statistical analysis was set at $p<.05$. SPSS for Windows version 19 (SPSS, Chicago, IL) was used for all analyses.

4. Results

4.1 Macrophage Characterization

The data indicates that SDR increased the number of *C. rodentium* could be colonized from the stool, with the infection peaking on day 12. The SDR group had a marginally significant increase in *C. rodentium* across the 24 day period ($F(1,64)=3.577$, $p=.064$), highest on day 12 post-challenge. Non-stressed HCC mice tended to show little or undetectable amounts of

C. rodentium (figure 1). The detectable range for this experiment was from log(10) 2.6 to log(10) 7.2 CFU/gram of fecal matter.

Figure 2 shows tissue samples after staining with F4/80 antibody. Positive cells shown on the left are visualized by a secondary antibody conjugated to horse radish peroxidase, which turns brown. Photoshop was used to convert positive brown cell pixels to black, and all other pixels to white. The percentage of black pixels in the area of the tissue was then determined using Photoshop. SDR significantly increased the percentage of $F4/80^{+}$ pixels in colon tissue over HCC over the course of the infection ($F(4,26)=3.12$, $p<.05$). Post-hoc testing indicated this was due to a significant increase on day 12 post-infection.

Figure 3 shows the results for real time PCR performed on colon tissue from stressed and non-stressed mice. Figures 3A and 3B show genes associated with M2 phenotypes, namely arginase and TGF-β. Although the mean level of TGF-β mRNA was lower in mice exposed to SDR compared to control mice, this difference did not reach statistical significance ($F(1,16=0.16$, not significant). Stressor exposure did not effect arginase mRNA levels ($F(1,16)$-0.59, not significant). However, as shown in graphs C, D, and E, expression levels of genes associated with M1 phenotypes appear to change with stress. TNF-α expression was found to be significantly increased in SDR over HCC across days ($F(3,24)=3.172$, $p<.05$) with significant differences occurring on day 6 post-infection. Expression of iNOS also significantly increases with stress across days ($F(3,24)=3.529$, $p<.05$) with SDR being higher than HCC on days 6, 12, and 24 post-challenge. However, CCL2 was not found to be significantly changed ($F(3,24)=9.337$, not significant).

Figure 4 shows the results of flow cytometry performed on the leukocytes isolated from the lamina propria of the colon. As seen in figures 4A and 4B, there are more $CCR2^{+}CD11b^{+}$

events in SDR mice than in HCC mice. Figure 4C shows that stress induces a marginally significant increase in $CD11b^+$ cells ($t(7)=2.23$, $p=.06$) and a marginally significant increase in double positive $CCR2^+CD11b^+$ cells ($t(6)=2.16$, $p=.07$). There was not a significant change in either $CD4^+$ cells ($t(4)=0.23$) or in $CD8^+$ cells ($t(4)=0.95$).

4.2 Azoxymethane Study

Figure 5 shows the levels of *C. rodentium* that were cultured from stool. The groups which received SDR, PCS (non-AOM **P**BS injection, ***C. rodentium*** infection, **S**DR) and ACS (AOM injection, *C. rodentium* infection, SDR) had a significantly increased colonization over the non-stressed groups, ACH (AOM injection, ***C. rodentium***, HCC) and PCH (non-AOM **P**BS injection, ***C. rodentium*** infection, HCC) ($F(3,64)=4.37$, $p<.05$) across days with highest colonization occurring on day 12. The PBS mice also had significantly higher colonization over the AOM mice ($F(1,64)=11.66$, $p<.001$)

Masses of spleen, liver, and colon were measured. There appeared to be no significant trend between any of the variables. The same seemed to be true for the length of the colon.

Table 2 shows histopathology scores assigned to colon tissue harvested from week 20 post-inejection. Tissues were assessed by a blinded pathologist for the listed inflammation pathology and given a score between 0 and 4, where 0 is healthy tissue and 4 is the most severe. There were no signs of continued inflammation in any of the samples, or indicators of tumors or pre-tumors.

5. Discussion

A previous study demonstrated that mice exposed to the SDR stressor during oral challenge with *C. rodentium* shed higher levels of *C. rodentium* into the stool and an increase in *C. rodentium*-induced pathology (Galley *et al.,* In Preparation). One measure in the pathogen colitis index is the accumulation of leukocytes in the colonic tissue, however, previously it was not known which types of leukocytes accumulate in the colon. The current study used immunohistochemistry for F4/80, to demonstrate that F4/80$^+$ cells increase with SDR and *C. rodentium* infection. Since F4/80 is generally a marker for macrophages, it is likely that it is F4/80$^+$ macrophages that are increasing with stress. However, dendritic cells (DC) can also display an F4/80$^+$ phenotype, so it is possible that cells in addition to macrophages increase in the colon during stressor exposure.

Working with the hypothesis that macrophages are increasing with stress, we sought to further characterize the macrophages. Since macrophages can be either pro-inflammatory M1 type or anti-inflammatory M2 type, we performed real time PCR on colon tissue from day 12 mice. We looked for gene expression associated with the two different phenotypes. Expression of TNF-α, and inducible nitric oxide synthase (iNOS), genes associated with M1 macrophages were increased, while arginase and TGF-β expression, genes associated with M2 macrophages, had no significant change. Although these cytokines can be made by other cell types they have been used as markers for characterization (Martinez, *et al.* 2008). Thus, it seems reasonable that the macrophage population increased in the colon is of the M1 phenotype.

To further characterize the colonic macrophage population, flow cytometry was performed on lymphocytes isolated from the lamina propria of the colon. It was shown that of CD45$^+$ cells (a marker for leukocytes), stress increased both CD11b$^+$ cells and double positive CCR2$^+$CD11b$^+$ cells. Other studies have also demonstrated increased populations of

$CCR2^{+}CD11b^{+}$ cells present in the colon during inflammation, and have found these cells to be cytokine producers (Bain, 2012) Flow analysis also revealed that $CD4^{+}$ and $CD8^{+}$ cells remained unchanged between stressed and unstressed animals, suggesting that increased T-cell numbers are not associated with stressor-enhanced inflammation.

Even though inflammatory macrophages and monocytes can be protective against bacterial infection (Fujiwara and Kobayashi, 2005), they can also contribute to uncontrolled colonic inflammation (Platt *et al.*, 2010). Platt *et al.* demonstrated that they could isolate two distinct macrophage populations in the gut, $F4/80^{+}TLR2^{-}CCR2^{-}CX3CR1^{hi}$ macrophages, which appear to be M2 type, and $F4/80^{+}TLR2^{+}CCR2^{+}CX3CR1^{int}$ macrophages, which appear to be M1 type. They isolated the M2 type macrophages from resting colons, and found that they produced little to no TNF-α upon LPS stimulation. They isolated M1 type macrophages from colons during experimental colitis, and found that they produced high levels of TNF-α upon stimulation. They also showed that CCR2 knockout mice showed reduced M1 type macrophages, and also reduced levels of colitis, suggesting that these macrophages are contributing to the inflammation. Conversely, Hunter *et al.* have shown that a tape worm *Hymenolepis dimunuta*, which has the ability to alternately activate macrophages, can reduce colitis in a DSS model. These alternately activated macrophages have increased expression levels of arginase and appear to be of the M2 phenotype, although further characterization was not performed (Hunter *et al.*, 2010).

Chronic inflammation is a well known predisposing factor for developing cancer (Coussens and Werb, 2002) (Perwez-Hussain and Harris, 2007), especially in the colon (Jawad *et al.*, 2011), because the inflammation creates an environment ideal for carcinogenesis. Enzymes such as iNOS produce free radicals which can induce mutations in host DNA. Inflammation is also often marked by an increase in growth factors, nutrients, and blood supply, all of which are

vital for the proliferation of cancer cells. Furthermore, colonic inflammation is often marked by rapid epithelial cell proliferation, a process which if mutated to become constitutive, could easily lead to tumor formation.

Stressor exposure increases *C. rodentium* induced pathology at the peak of infection (Galley *et al.,* In Preparation). In this study, we did not find evidence that the increased inflammation increased susceptibility to development of CRC. In fact, tumors or pre-tumor histological activity was not found in any animal in this study. It is important to note however, that the chronicity of inflammation is an important factor for CRC development. In our model, we know that stressor-enhanced colonic inflammation peaks around day 12 post-pathogen, and that both infection and inflammation is essentially resolved by day 24 post-infection (Galley *et al.,* Unpublished Observation). Thus, it is possible that a more prolonged inflammatory response would be more likely to induce CRC development.

However, this study was based off of studies by Suzuki *et al.*, which used dextran sodium sulfate (DSS) as the mode for inducing colonic inflammation. Suzuki's lab demonstrated that CRC could be induced with a single injection of AOM followed a week later by a week of DSS administration, whereas previous labs used multiple weekly injections of AOM (Suzuki *et al*., 2003). Our study simply replaced the DSS step with *C. rodentium* infection and SDR. DSS induces a similar, albeit more severe, inflammatory response in the colon, and resolves itself in a similar time frame (Yan *et al.,* 2009). Since the inflammation induced by DSS lasts for a similar time frame to our lab's model, it seems less likely that a short duration of inflammation was the responsible for a lack of tumor formation. It is also possible that Suzuki's model is effective because DSS induces a more severe and disruptive colitis than our model.

Furthermore, our results show that mice that received a PBS IP injection had significantly higher *C. rodentium* colonizations over mice that received AOM. Thus, it is possible that AOM could have either interfered with *C. rodentium* colonization, or killed *C. rodentium* directly directly, leading to even less severe colitis.

Since CRC can be induced with AOM injections alone, it is possible that SDR still might increase susceptibility for CRC if more than one AOM injection was administered. It is also possible that a different form of stressor used by this lab, namely prolonged restraint, could also prove more successful, due to the more severe colitis it illicits.

Conclusion:

This study demonstrated that F4/80^{+} cells increase in the colon with social stress and pathogen challenge and that these macrophages are likely of the M1 phenotype. Gene expression levels that are associated with and are used to mark M1 macrophages are increased in stressed mice over non-stressed mice. Furthermore, flow analysis showed that these cells had markers commonly accepted as markers of inflammatory macrophages. Additional studies are needed to further confirm these results.

Figure Legends

Figure 1: *C. rodentium* colonization during SDR. Fecal sheddings were collected on days 1, 3, 6, 12, and 24 post-infection, homogenized and plated on Lactose Mackoney agar supplemented with kanamycin (40 μg/ml) at 37ºC for 24 hrs. These data points represent the log(10) colony of forming units (CFU) per gram of fecal matter +/- standard error mean (SEM). The SDR group had a marginally significant increase in *C. rodentium* across the 24 day period ($F(1,64)=3.577$, $p=.064$), highest on day 12 post-challenge. n=6

Figure 2: F4/80$^+$ staining of colonic tissue during SDR. Colon tissue was harvested from SDR and HCC mice on days 0, 3, 6, 12, and 24 post-challenge with *C. rodentium*, fixed in 10% formalin, embedded in paraffin. Figure 2A and 2C represent colon tissue stained with F4/80 primary antibodies and visualized with horse radish peroxidase secondary antibodies. F4/80$^+$ cells are seen as brown. Samples were photographed at 100X and quantified using Photoshop. Positive pixels were turned black and negative white with a histogram in order to determine percentage of positive pixels, as shown in Figures 2B and 2C. Data points in Figure 2E represent mean F4/80$^+$ pixel percentage +/- SEM. SDR significantly increased the percentage of F4/80$^+$ pixels in colon tissue over HCC over the course of the infection ($F(4,26)=3.12$, $p<.05$). Post-hoc testing indicated this was due to a significant increase on day 12 post-infection. Day 12, n=6, day 0, 3, 6, 24 n=3

Figure 3: Real Time PCR of mRNA isolated from colonic tissue during SDR. Colon tissue was harvest from mice on days 0, 6, 12, and 24 post-challenge with *C. rodentium*, mRNA was isolated using Trizol, and cDNA was synthesized. Real time PCR was run using mRNA primers for TGF-β, ARG-1, TNF-α, iNOS, and CCL2. Data points on Figures 3A-3E represent mean n-fold increase of expression over control mice minus expression of housekeeping gene 18S +/-

SEM. Figure 3A shows the mean level of TGF-β mRNA was lower in mice exposed to SDR compared to control mice, but was not statistical significance ($F(1,16=0.16$, not significant). Figure 3B shows stressor exposure did not effect arginase mRNA levels (F(1,16)-0.59, not significant). In Figure 3C shows TNF-α significantly increased in SDR over HCC group across days ($F(3,24)=3.172$, $p<.05$) with significant differences occurring on day 6 post-infection. Figure 2D shows expression of iNOS also significantly increases with stress across days ($F(3,24)=3.529$, $p<.05$) with SDR being higher than HCC on days 6, 12, and 24 post-challenge. Figure 2E shows CCL2 did not significantly change ($F(3,24)=9.337$, not significant). n=3

Figure 4: Flow cytometry of day 12 colon tissue. Colons from mice subjected to SDR and HCC were harvested; lamina proprial lymphocytes were isolated and stained with stained with CD45-FITC, F4/80-PerCP, and CD11b-APC in panel one and CD45-FITC, CCR2-PE, and CD11b-APC in panel two. Two panels for lymphocytes were also used, with antibodies for CD45-FITC, CD3-PE, CD4-PerCP and CD8-APC. Figures 4A and 4B show that there are more $CCR2^{+}CD11b^{+}$ events in SDR mice than in HCC mice. Figure 4C shows that SDR mice had a marginally significant increase in $CD11b^{+}$ cells ($t(7)=2.23$, $p=.06$) and 4D shows a marginally significant increase in double positive $CCR2^{+}CD11b^{+}$ cells ($t(6)=2.16$, $p=.07$). Figures 4E and 4F show there was not a significant change in either $CD4^{+}$ cells ($t(4)=0.23$) or in $CD8^{+}$ cells ($t(4)=0.95$). n=3

Figure 5: Figure 1: *C. rodentium* colonization during SDR and AOM. Fecal sheddings were collected on days 1, 3, 6, 12, and 24 post-infection, homogenized and plated on Lactose Mackoney agar supplemented with kanamycin (40 μg/ml) at 37ºC for 24 hrs. These data points represent the log(10) colony of forming units (CFU) per gram of fecal matter +/- SEM. The groups which received SDR, and ACS had a significantly increased colonization over the non-

stressed groups, ACH and PCH ($F(3,64)=4.37$, $p<.05$) across days with highest colonization occurring on day 12. The PBS mice also had significantly higher colonization over the AOM mice ($F(1,64)=11.66$, $p<.001$). n=6

Figure 7: Organ masses and lengths at 20 weeks post-injection. Mice were euthanized and their livers, spleens, and colons were removed and weighed. Colon length was measured. There were no statistically significant differences in group for spleen mass ($F(1,40)=.446$) not significant), liver mass ($F(1,40)=3.895$), colon mass ($F(1,45)=0.04$), or colon length ($F(1,40)=1.622$). n=6

Table 1: Pathology Assessment of AOM Colon Tissue. Colon tissue was harvest from mice 20 weeks post-injection, fixed in formalin, embedded in paraffin, stained with H&E, and scored by a pathologist based on a scale of 0 to 4, 0 being healthy. No changes were observed.

Figures and Tables

Figure 1

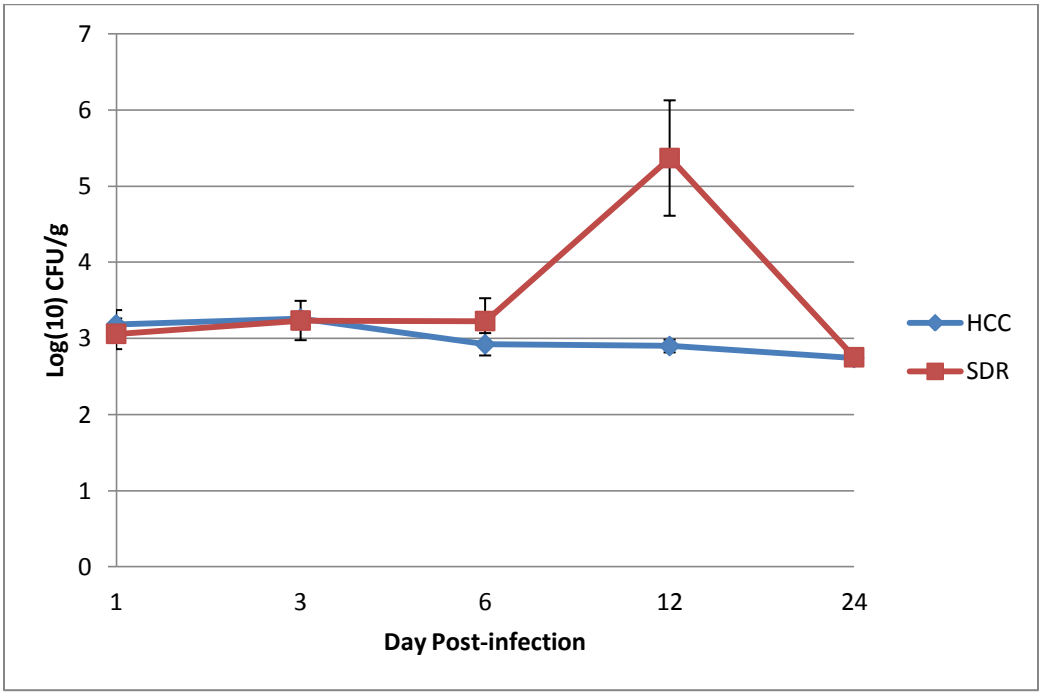
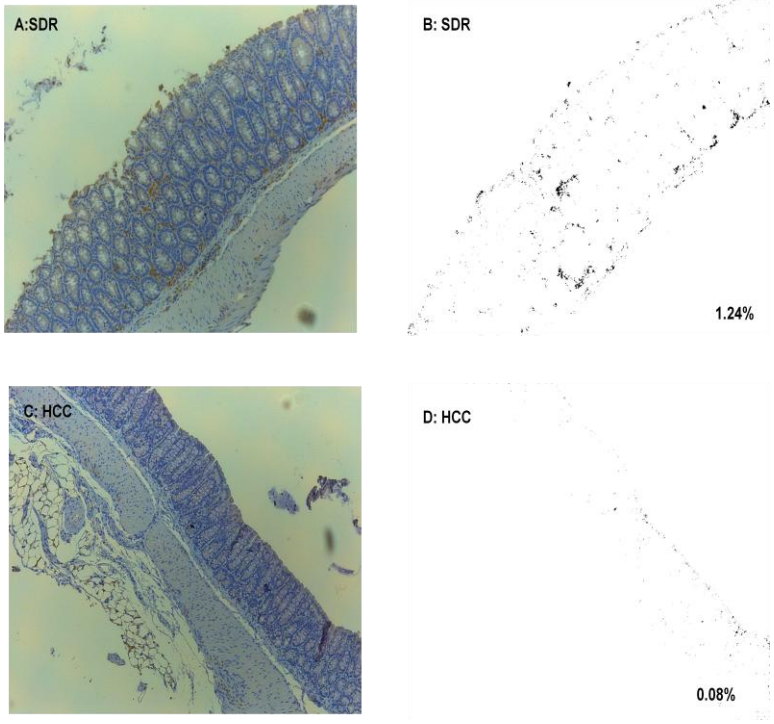


Figure 2


A:SDR
B: SDR
1.24%
C: HCC
D: HCC
0.08%

D:

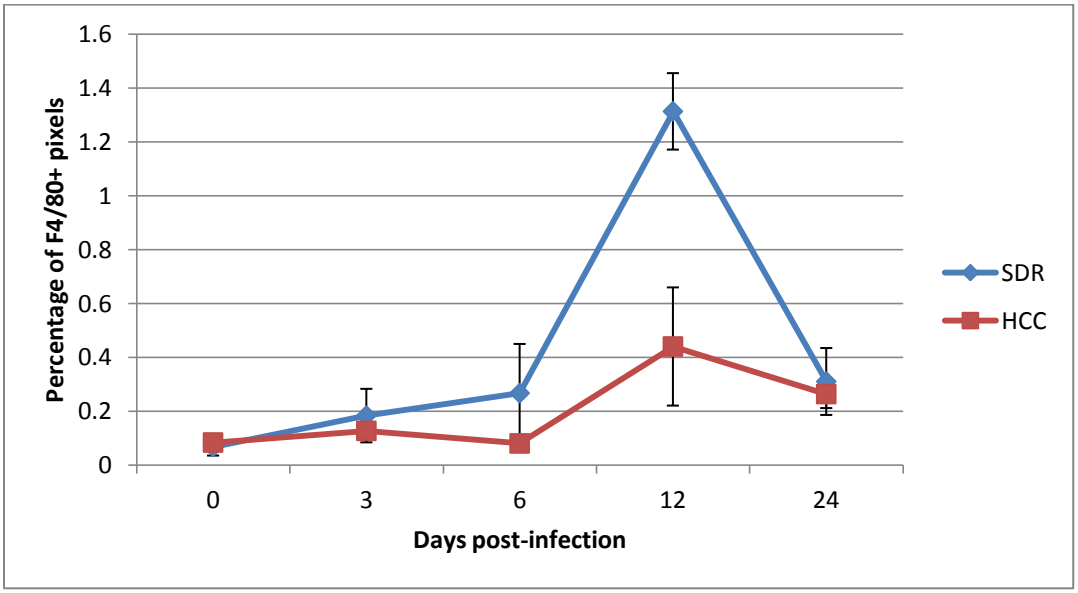
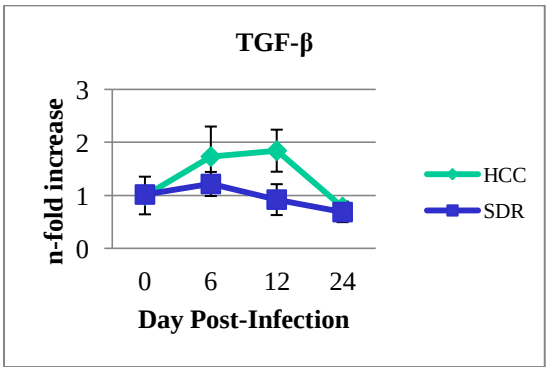
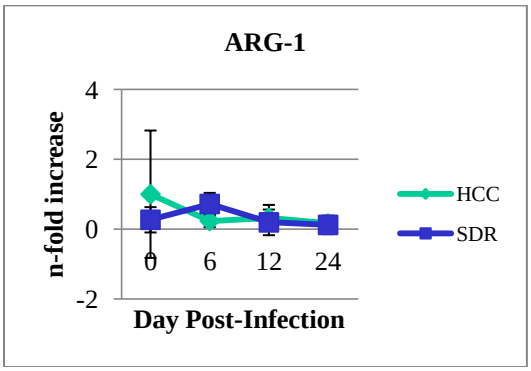

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Percentage of F4/80+ pixels
0
3
6
12
24
Days post-infection
SDR
HCC

Figure 3

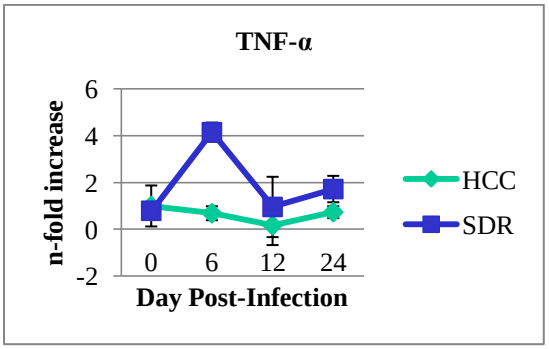
A:



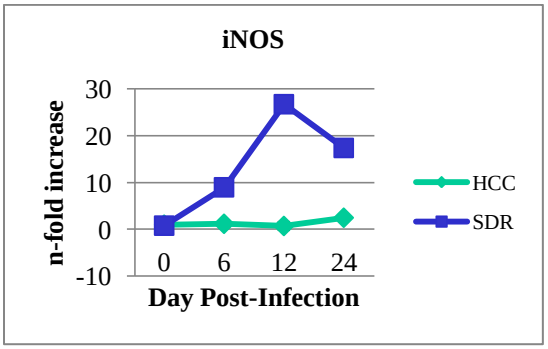
B:



C:



D:



E:

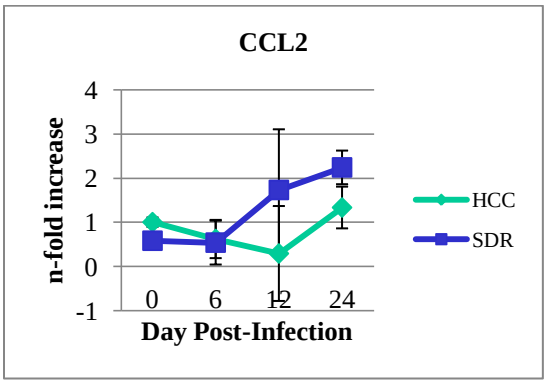
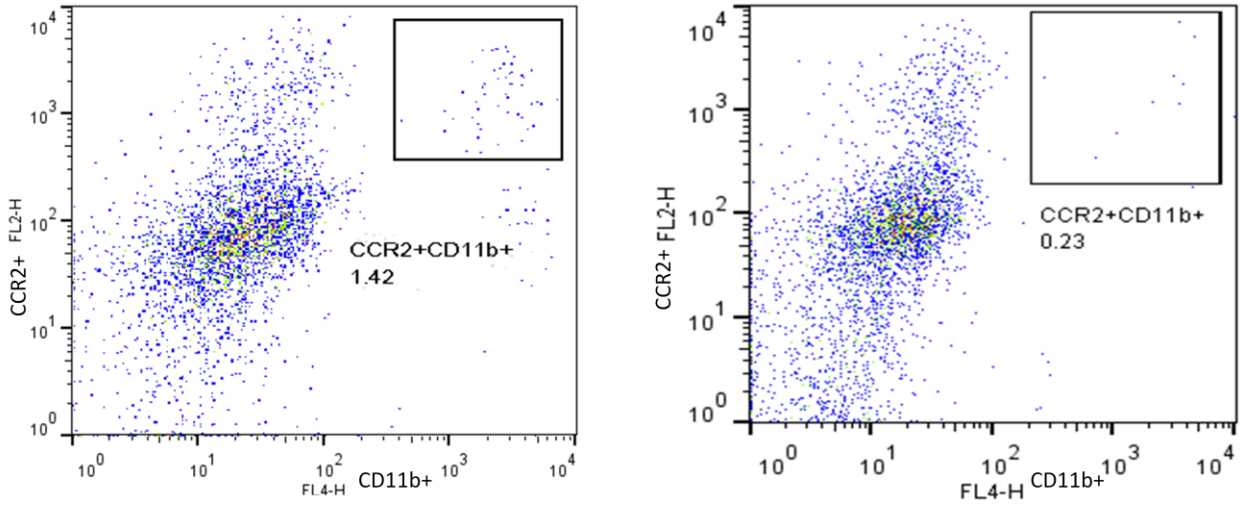
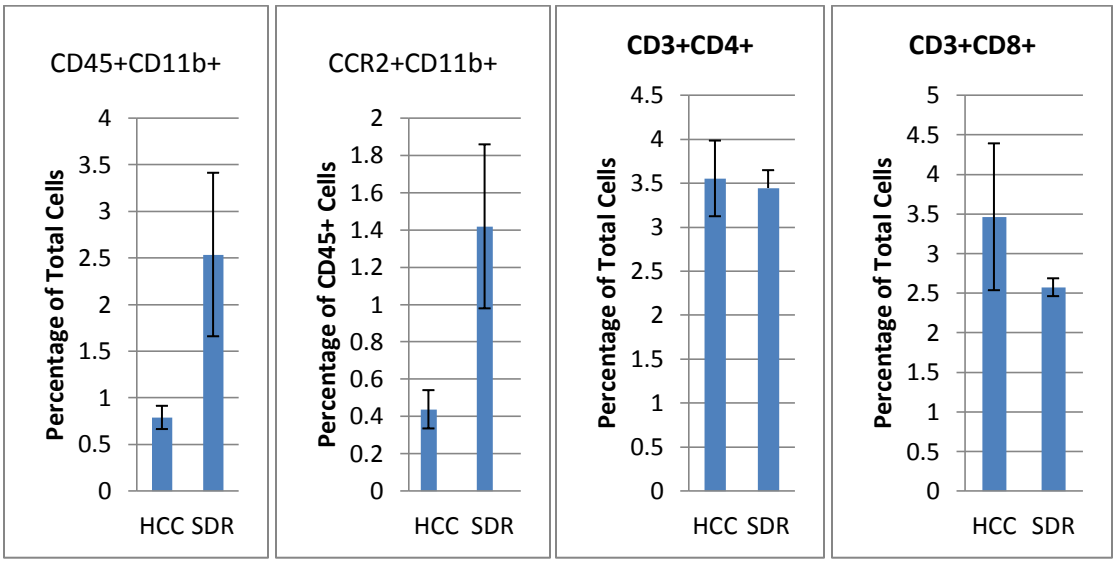


Figure 4



C: D: E: F:



AOM group:

C.rodentium + SDR (ACS)	Vehicle + SDR (AVS)
C.rodentium + HCC (ACH)	Vehicle + HCC (AHS)

PBS group:

C.rodentium + SDR (PCS)	Vehicle + SDR (PVS)
C.rodentium + SDR (PCH)	Vehicle + HCC (PHS)

Figure 5

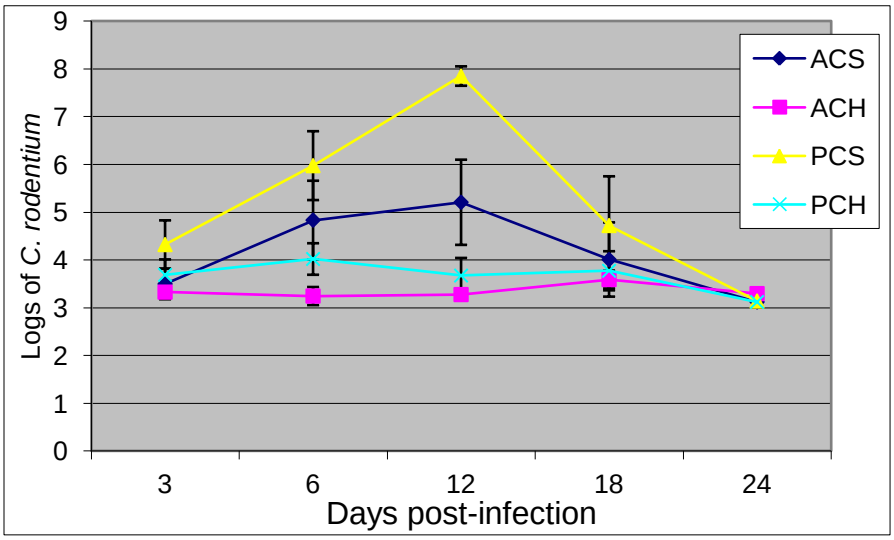


Figure 6

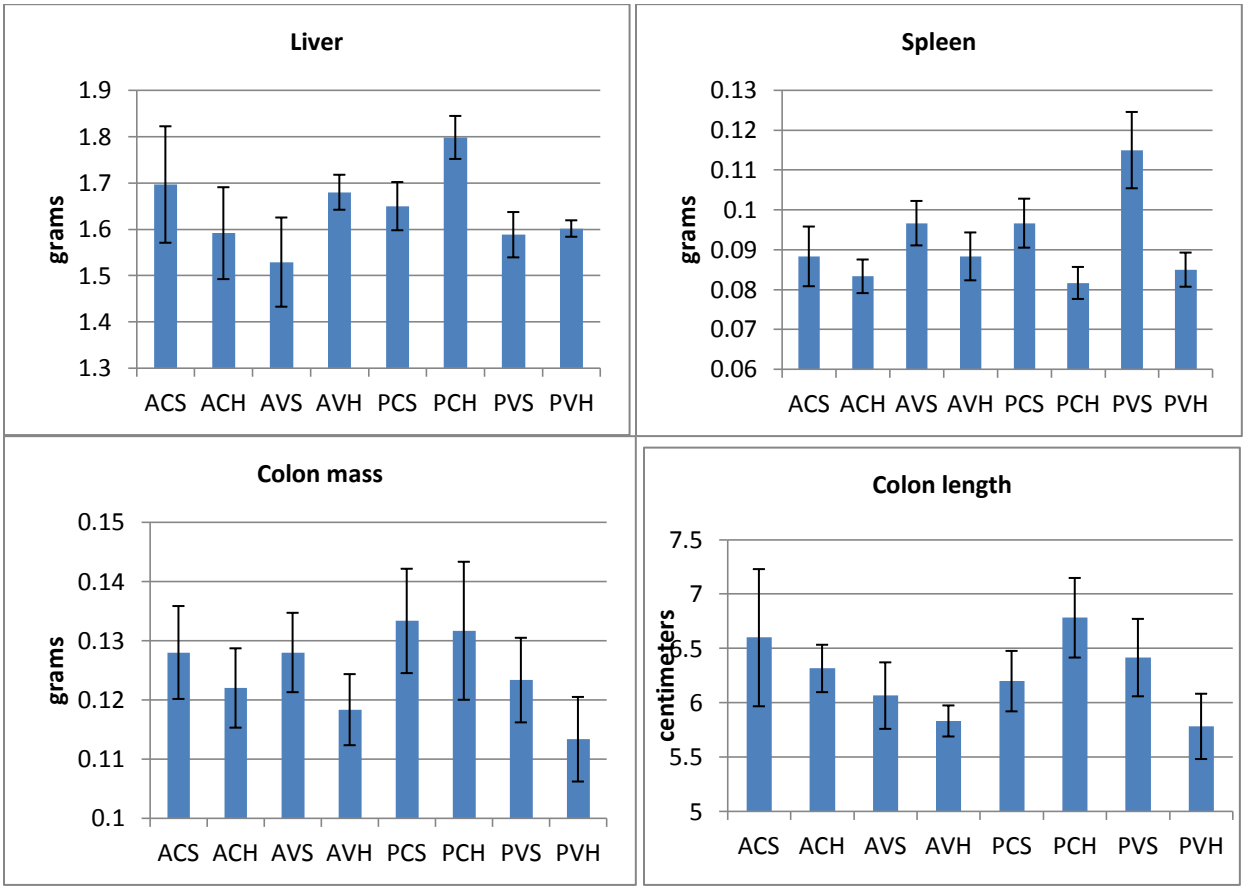

Liver
grams
1.9
1.8
1.7
1.6
1.5
1.4
1.3
ACS ACH AVS AVH PCS PCH PVS PVH
Spleen
grams
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0.06
ACS ACH AVS AVH PCS PCH PVS PVH
Colon mass
grams
0.15
0.14
0.13
0.12
0.11
0.1
ACS ACH AVS AVH PCS PCH PVS PVH
Colon length
centimeters
7.5
7
6.5
6
5.5
5
ACS ACH AVS AVH PCS PCH PVS PVH

Table 1

	Inflamm	Edema	Epithelial defects	Crypt atrophy	Hyperplasia	Dysplasia
PCS 1	0	0	0	0	0	0
PCS 2	0	0	0	0	0	0
PCS 3	0	0	0	0	0	0
PCH 1	0	0	0	0	0	0
PCH 2	0.5	0	0	0	0	0
PCH 3	0	0	0	0	0	0
ACH 4	0	0	0	0	0	0
ACH 5	0	0	0	0	0	0
ACH 6	0	0	0	0	0	0
ACS 1	0.5	0	0	0	0	0
ACS 2	0	0	0	0	0	0
ACS 3	0.5	0	0	0	0.5	0

References

Abraham, C., Cho, J.H., 2009. Inflammatory Bowel Disease. N. Engl. J. Med. 361, 2066-2078

Ambs, S., Merriam, W.G., Bennett, W.P., Felley-Bosco, E., Ogunfusika, M.O., Oser, S.M., Klein, S., Shields, P.G., Billiar, T.R., Harris, C.C., 1998. Frequent Nitric Oxide Synthase-2 Expression in Human Colon Adenomas: Implication for Tumor Angiogenesis and Colon Cancer Progression. Cancer Research. 58, 334-341

Avitsur, R., Stark, J.L., Sheridan, J.F., 2001. Social Stress Induces Glucocorticoid Resistance in Subordinate Animals. Horm. Behav. 39, 247-257

Bachmann, M.F., Kopf, M., Marsland, B.J., 2006. Chemokines: more than just road signs. Nat Rev Immunol. 6, 159-164

Bain, C.C., Scott, C.L., Uronen-Hansson, H., Gudjonsson, S., Jansson, O., Grip, O., Guilliams, M., Malissen, B., Agace, W.W., Mowat, A.M., 2012. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. Mucosal Immunol

Bartolomucci, A., 2007. Social stress, immune functions and disease in rodents. Front. Neuroendocrinol. 28, 28-49

Bhinder, G., Sham, H.P., Chan, J.M., Morampudi, V., Jacobson, K., Vallance, B.A., 2013. The Citrobacter rodentium Mouse Model: Studying Pathogen and Host Contributions to Infectious Colitis. J Vis Exp, e50222

Borrello, M.G., Alberti, L., Fischer, A., Degl'Innocenti, D., Ferrario, C., Gariboldi, M., Marchesi, F., Allavena, P., Greco, A., Collini, P., Pilotti, S., Cassinelli, G., Bressan, P., Fugazzola, L., Mantovani, A., Pierotti, M.A., 2005. Induction of a proinflammatory program in normal human thyrocytes by the RET/PTC1 oncogene. Proceedings of the National Academy of Sciences of the United States of America. 102, 14825-14830

Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A., Schirle, M., Schlegl, J., Schwab, M., Stein, M.A., Bauer, A., Casari, G., Drewes, G., Gavin, A., Jackson, D.B., Joberty, G., Neubauer, G., Rick, J., Kuster, B., Superti-Furga, G., 2004. A physical and functional map of the human TNF-alpha]/NF-kappa]B signal transduction pathway. Nat. Cell Biol. 6, 97-105

Carrasco, G.A., Van de Kar, L.D., 2003. Neuroendocrine pharmacology of stress. Eur. J. Pharmacol. 463, 235-272

Chan, A.T., Ogino, S., Fuchs, C.S., 2007. Aspirin and the Risk of Colorectal Cancer in Relation to the Expression of COX-2. N. Engl. J. Med. 356, 2131-2142

Colotta, F., Allavena, P., Sica, A., Garlanda, C., Mantovani, A., 2009. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis. 30, 1073-1081

Costanzo, E.S., Sood, A.K., Lutgendorf, S.K., 2011. Biobehavioral Influences on Cancer Progression. Immunology and Allergy Clinics of North America. 31, 109-132

Coussens, L.M., Werb, Z., 2002. Inflammation and cancer. Nature. 420, 860-867

Coutinho, A.E., Chapman, K.E., 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol. Cell. Endocrinol. 335, 2-13

DA SILVA, J.A.P., 1999. Sex Hormones and Glucocorticoids: Interactions with the Immune System. Ann. N. Y. Acad. Sci. 876, 102-118

DALY, C., ROLLINS, B.J., 2003. Monocyte Chemoattractant Protein-1 (CCL2) in Inflammatory Disease and Adaptive Immunity: Therapeutic Opportunities and Controversies. Microcirculation. 10, 247-257

Dhabhar, F.S., 2002. Stress-induced augmentation of immune function—The role of stress hormones, leukocyte trafficking, and cytokines. Brain Behav. Immun. 16, 785-798

Dhabhar, F.S., Malarkey, W.B., Neri, E., McEwen, B.S., 2012. Stress-induced redistribution of immune cells—From barracks to boulevards to battlefields: A tale of three hormones – Curt Richter Award Winner. Psychoneuroendocrinology. 37, 1345-1368

Dinarello, C.A., 2009. Interleukin-1β and the Autoinflammatory Diseases. N. Engl. J. Med. 360, 2467-2470

Engler, H., Bailey, M.T., Engler, A., Sheridan, J.F., 2004. Effects of repeated social stress on leukocyte distribution in bone marrow, peripheral blood and spleen. J. Neuroimmunol. 148, 106-115

Eubank, T.D., Roberts, R.D., Khan, M., Curry, J.M., Nuovo, G.J., Kuppusamy, P., Marsh, C.B., 2009. Granulocyte Macrophage Colony-Stimulating Factor Inhibits Breast Cancer Growth and Metastasis by Invoking an Anti-Angiogenic Program in Tumor-Educated Macrophages. Cancer Research. 69, 2133-2140

Fujiwara, N., Kobayashi, K., 2005. Macrophages in inflammation. Curr Drug Targets Inflamm Allergy. 4, 281-286

Giese-Davis, J., Spiegel, D., 2003. Emotional expression and cancer progression, in: Handbook of affective sciences. New York, NY, US: Oxford University Press, pp. 1053-1082

Gordon, S., Hamann, J., Lin, H., Stacey, M., 2011. F4/80 and the related adhesion-GPCRs. Eur. J. Immunol. 41, 2472-2476

Gordon, S., Taylor, P.R., 2005. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 5, 953-964

Hanauer, S.B., Feagan, B.G., Lichtenstein, G.R., Mayer, L.F., Schreiber, S., Colombel, J.F., Rachmilewitz, D., Wolf, D.C., Olson, A., Bao, W., Rutgeerts, P., 2002. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. The Lancet. 359, 1541-1549

Higgins, L.M., Frankel, G., Douce, G., Dougan, G., MacDonald, T.T., 1999. Citrobacter rodentium Infection in Mice Elicits a Mucosal Th1 Cytokine Response and Lesions Similar to Those in Murine Inflammatory Bowel Disease. Infection and Immunity. 67, 3031-3039

Hunter, M.M., Wang, A., Parhar, K.S., Johnston, M.J.G., Van Rooijen, N., Beck, P.L., McKay, D.M., 2010. In Vitro-Derived Alternatively Activated Macrophages Reduce Colonic Inflammation in Mice. Gastroenterology. 138, 1395-1405

Jawad, N., Direkze, N., Leedham, S.J., 2011. **Inflammatory Bowel Disease and Colon Cancer**. Recent Results in Cancer Research. 185, 99-115

Jung, Y., Isaacs, J.S., Lee, S., Trepel, J., Neckers, L., 2003. IL-1β mediated up-regulation of HIF-1α via an NFkB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. The FASEB Journal

Khani, S., Tayek, J.A., 2001. Cortisol increases gluconeogenesis in humans: its role in the metabolic syndrome. Clin. Sci. 101, 739-747

Koshiji, M., To, K.K.-., Hammer, S., Kumamoto, K., Harris, A.L., Modrich, P., Huang, L.E., 2005. HIF-1α Induces Genetic Instability by Transcriptionally Downregulating MutSα Expression. Mol. Cell. 17, 793-803

Kuziel, W.A., Morgan, S.J., Dawson, T.C., Griffin, S., Smithies, O., Ley, K., Maeda, N., 1997. Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2. Proceedings of the National Academy of Sciences. 94, 12053-12058

Lakatos, P.L., Lakatos, L., 2008. **Risk for colorectal cancer in ulcerative colitis: Changes, causes and management strategies**. World J Gastroenterol. 14, 3937–3947

Leonard, B.E., 2005. The HPA and immune axes in stress: The involvement of the serotonergic system. European Psychiatry. 20, Supplement 3, S302-S306

Locksley, R.M., Killeen, N., Lenardo, M.J., 2001. The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology. Cell. 104, 487-501

Lutgendorf, S.K., Sood, A.K., November-December 2011. Biobehavioral Factors and Cancer Progression: Physiological Pathways and Mechanisms. Psychosomatic Medicine. 73, 724-730

Martinez, F., Sica, A., Mantovani, A., Locati, M., 2008. Macrophage activation and polarization. Front. Biosci. 13, 453-461

Miyazaki, K., Itoh, N., Ohyama, S., Kadota, K., Oishi, K., 2013. Continuous exposure to a novel stressor based on water aversion induces abnormal circadian locomotor rhythms and sleep-wake cycles in mice. PLoS One. 8, e55452

Nance, D.M., Sanders, V.M., 2007. Autonomic innervation and regulation of the immune system (1987-2007). Brain Behav. Immun. 21, 736-745

Perwez Hussain, S., Harris, C.C., 2007. Inflammation and cancer: An ancient link with novel potentials. International Journal of Cancer. 121, 2373-2380

Platt, A.M., Bain, C.C., Bordon, Y., Sester, D.P., Mowat, A.M., 2010. An Independent Subset of TLR Expressing CCR2-Dependent Macrophages Promotes Colonic Inflammation. The Journal of Immunology. 184, 6843-6854

Pruett, S.B., 2003. Stress and the immune system. Pathophysiology. 9, 133-153

Reiche, E.M.V., Nunes, S.O.V., Morimoto, H.K., 2004. Stress, depression, the immune system, and cancer. The Lancet Oncology. 5, 617-625

Rustgi, A.K., 2007. The genetics of hereditary colon cancer. Genes & Development. 21, 2525-2538

Santos, J., Yang, P., Söderholm, J.D., Benjamin, M., Perdue, M.H., 2001. Role of mast cells in chronic stress induced colonic epithelial barrier dysfunction in the rat. Gut. 48, 630-636

Saul, A.N., Oberyszyn, T.M., Daugherty, C., Kusewitt, D., Jones, S., Jewell, S., Malarkey, W.B., Lehman, A., Lemeshow, S., Dhabhar, F.S., 7 December 2005. Chronic Stress and Susceptibility to Skin Cancer. Journal of the National Cancer Institute. 97, 1760-1767

Schauer, D.B., Zabel, B.A., Pedraza, I.F., O'Hara, C.M., Steigerwalt, A.G., Brenner, D.J., 1995. Genetic and biochemical characterization of Citrobacter rodentium sp. nov. Journal of Clinical Microbiology. 33, 2064-2068

Segerstrom, S.C., Miller, G.E., 2004. Psychological Stress and the Human Immune System: A Meta-Analytic Study of 30 Years of Inquiry. Psychol. Bull. 130, 601-630

Shachar, I., Karin, N., 2013. The dual roles of inflammatory cytokines and chemokines in the regulation of autoimmune diseases and their clinical implications. Journal of Leukocyte Biology. 93, 51-61

Sica, A., Allavena, P., Mantovani, A., 2008. Cancer related inflammation: The macrophage connection. Cancer Lett. 267, 204-215

Sloan, E.K., Priceman, S.J., Cox, B.F., Yu, S., Pimentel, M.A., Tangkanangnukul, V., Arevalo, J.M.G., Morizono, K., Karanikolas, B.D.W., Wu, L., Sood, A.K., Cole, S.W., 2010. The Sympathetic Nervous System Induces a Metastatic Switch in Primary Breast Cancer. Cancer Research

Szlosarek, P., Charles, K.A., Balkwill, F.R., 2006. Tumour necrosis factor-α as a tumour promoter. Eur. J. Cancer. 42, 745-750

Takahashi, M., Kitahashi, T., Ishigamori, R., Mutoh, M., Komiya, M., Sato, H., Kamanaka, Y., Naka, M., Maruyama, T., Sugimura, T., Wakabayashi, K., 2008. Increased expression of inducible nitric oxide synthase (iNOS) in N-nitrosobis(2-oxopropyl)amine-induced hamster pancreatic carcinogenesis and prevention of cancer development by ONO-1714, an iNOS inhibitor. Carcinogenesis. 29, 1608-1613

Takahashi, M., Mutoh, M., Shoji, Y., Sato, H., Kamanaka, Y., Naka, M., Maruyama, T., Sugimura, T., Wakabayashi, K., 2006. Suppressive effect of an inducible nitric oxide inhibitor, ONO-1714, on AOM-induced rat colon carcinogenesis. Nitric Oxide. 14, 130-136

Tanaka, T., Kohno, H., Suzuki, R., Yamada, Y., Sugie, S., Mori, H., 2003. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Science. 94, 965-973

Terzić, J., Grivennikov, S., Karin, E., Karin, M., 2010. Inflammation and Colon Cancer. Gastroenterology. 138, 2101-2114.e5

Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F., Cerami, A., 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature. 330, 662-664

Venzala, E., García-García, A.L., Elizalde, N., Delagrange, P., Tordera, R.M., 2012. Chronic social defeat stress model: behavioral features, antidepressant action, and interaction with biological risk factors. Psychopharmacology (Berl.). 224, 313-325

Vincent, F.B., Morand, E.F., Murphy, K., Mackay, F., Mariette, X., Marcelli, C., 2013. Antidrug antibodies (ADAb) to tumour necrosis factor (TNF)-specific neutralising

agents in chronic inflammatory diseases: a real issue, a clinical perspective. Annals of the Rheumatic Diseases. 72, 165-178

Walburn, J., Vedhara, K., Hankins, M., Rixon, L., Weinman, J., 2009. Psychological stress and wound healing in humans: A systematic review and meta-analysis. J. Psychosom. Res. 67, 253-271

Yan, Y., Kolachala, V., Dalmasso, G., Nguyen, H., Laroui, H., Sitaraman, S.V., Merlin, D., 2009. Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis. PLoS One. 4, e6073